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PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Hiroaki TAKAYAMA, et al.

Appln. No.: 09/214,155 Group Art Unit: 1616

Filed: December 29,1998 Examiner: Sabiha N. Qazi

For: VITAMIN D3 DERIVATIVE AND ITS PRODUCTION METHOD

FUJISHIMA DECLARATION UNDER 37 C.F.R. § 1.131

Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Toshie Fujishima, hereby declare and state:

THAT I am a citizen of Japan;

THAT I received a Masters Degree in 1993 and a Ph.D. degree in 1996 from Tokyo University;

THAT I am a member of several Japanese Scientific Societies related to research in organic chemistry and Vitamin D;

THAT I have been employed by Teikyo University, Faculty of Pharmaceutical Sciences since 1996, where I have been involved in the synthetic study of Vitamin D_3 in Professor TAKAYAMA's group.

I am one of the joint inventors of the invention claimed in the above-identified application.

I have a thorough knowledge of the invention in the above-identified patent application, and I have read the non-final Office Action of June 10, 2002 issued in reference to the application. In response to the non-final Office Action, I submit herewith this Declaration, which explains that I am the author of the experimental note of certain compounds claimed in the application and which explains my role in inventing the subject matter of the claims of the application.

Author of experimental notes

I am the author of experimental notes (1), (2), and (3). (*see* Exhibit 1).

My role in the invention claimed in the application

I synthesized compound (68) (Example 2, page 33 of the specification) prior to March 17, 1997, as described in experimental note (1) and compound (72) (Example 1, page 32 of the specification) prior to March 17, 1997, as described in experimental note (2). I ordered a NMR analysis of compound (68) and compound (72) from Instrument Analysis Center of Teikyo University, Faculty of Pharmaceutical Sciences, and the analysis was carried out prior to March 17, 1997 by Ms. Junko Shimode, a NMR operator at the center. Corresponding NMR spectra are shown in Exhibit 1, Chart 1 (Compound (68)) and Chart 2 (Compound (72)). I reviewed the NMR spectra prior to March 17, 1997, and I identified the compounds as those compounds designated compound (68) and compound (72) in the present application. I measured the Vitamin D receptor (VDR) affinity of compound (68) and compound (72) prior to March 17, 1997, as described in experimental note (3). Corresponding data from a liquid scintillation counter is shown in Exhibit 1, Chart 3. I presented the results of the work to my colleagues in one of Professor TAKAYAMA's group seminars prior to March 17, 1997. (*see* Exhibit 2)

Prior to March 17, 1997, I also submitted an abstract for a poster session at the Tenth Workshop on Vitamin D. (*see* Exhibit 3)

These activities are described in detail below with reference to the notebook pages and actual data. Because the text in my notebook and on the data is in Japanese, I have also included copies of the pages and data with English text added.

Exhibit 1 is a compilation of data and pages from my notebook number 2, which I kept in the ordinary course of my research. The entries are in my handwriting.

Note 1 is dated in the upper left-hand corner. The date is prior to March 17, 1997. This page shows synthesis of compound #346 from the protected precursor compound #345. Compound #346 is designated compound (68) in the above-identified application.

The upper left corner of the page also has the notation "#346." This means that I planned to make compound #346. The structure of synthesized compound #346 is shown in the upper right of the page. The structure is the same as that for the compound designated compound (68) in the specification of the above-identified application. The compound shown in the upper left of the page is the protected precursor. "TBS" stands for tert-butyldimethylsilyl. The arrow pointing from protected precursor compound #345 to synthesized compound #346 indicates that I planned to make synthesized compound #346 from protected precursor compound #345.

Below the structural formulas in a bracket is the notation "#345 work up," which means that I used compound #345 as a starting compound. I dissolved compound #345 in 1 ml of methanol (MeOH) and then added 11mg of CSA (Camphor sulfonic acid). The notations "20:30~" and "11:00," which appear to the right side of the open bracket mean that the reaction mixture was stirred from 20:30 (eight thirty PM), to 11:00 (eleven o'clock). The dates, which have been blocked out, indicate that the stirring time was about 38 ½ hours.

Below the open bracket, I indicate that the mixture was stirred at room temperature (rt) and then the methanol was evaporated from the reaction mixture. To the residue I added water and then extracted with EA (ethyl acetate). The extract was washed with brine, dried over $MgSO_4$, filtered, and evaporated to give compound #346.

Below this description I indicate that the crude product was purified by silica gel chromatography and further by HPLC (ODS (18)). I then ordered a ^1H NMR analysis of the compound.

Chart 1 is dated prior to March 17, 1997. Chart 1 is a ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound #346 i.e. compound (68) and its numerically outputted data. This data was assigned as compound #346 as follows: δ 0.55 (3 H, s), 0.85 (3 H, d, J = 6.4 Hz), 1.15 (3 H, d, J = 6.7 Hz), 1.21 (6 H, s), 2.42 (1 H, dd, J = 13.9, 4.9 Hz), 2.52 (1 H, br. d, J = 13.9 Hz), 2.82 (1 H, dd, J = 11.9, 4.0 Hz), 4.02 (2 H, m), 5.02 (1 H, t, J = 1.8 Hz), 5.37 (1 H, t, J = 1.8 Hz), 6.03 (1 H, d, J = 11.3 Hz), 6.35 (1 H, d, J = 11.3 Hz).

Note 2 is dated in the upper left-hand corner. The date is prior to March 17, 1997. This page shows synthesis of compound #344 from the protected precursor compound #343. Compound #344 is designated compound (72) in the above-identified application.

The upper left corner of the page also has the notation "#344." This means that I planned to make compound #344. The structure of compound #344 is shown in the upper right of the page. The structure is the same as that for the compound designated compound (72) in the specification of the above-identified application. The compound shown in the upper left of the page is the protected precursor. "TBS" stands for tert-butyldimethylsilyl. The arrow pointing from compound #344 to compound #343 indicates that I planned to make compound #344 from compound #343.

In a bracket below the structural formulas is the notation "#343 work up," which means that I used compound #343 as a starting compound. I dissolved compound #343 in 1 ml of methanol (MeOH) and then added 11 mg of CSA (camphor sulfonic acid).

Below the open bracket, I indicate that the mixture was stirred from 14:20 (two twenty PM) to 9:00 (nine o'clock), the next day under Ar gas at room temperature (rt), and then the methanol was distilled away from the reaction mixture. To the residue I added water and then extracted with EA (ethyl acetate). The extract was washed with brine, dried over $MgSO_4$, dehydrated, filtered, and evaporated to give compound #344.

Below this description I indicate that the crude product was purified by silica gel chromatography and further by HPLC (ODS (18)). "ϕ 0.9 cm 10 cm height" means silica gel column size (0.9 cm diameter and 10 cm length). "EA : n-hex = 1:1" indicates the solvent system for the chromatography (ethyl acetate : n-hexane = 1 : 1).

Drawings at the bottom of the page are illustrations of TLC (thin layer chromatography). "EA : n-hex = 1:1" means a solvent system of the TLC (ethyl acetate : n-hexane = 1 : 1). "SM" and "RM" mean starting material and reaction mixture. Circles in the illustrations show spots on TLC. TLC is a typical monitor system of organic reaction because the spots show compound(s) in analyzed material. A position difference between the spot labeled "SM" and the spot labeled "RM" means that the compound(s) in the "SM" and "RM" spots were different and the reaction was complete.

I then ordered a ^{1}H NMR analysis of the compound.

Chart 2 is dated prior to March 17, 1997. Chart 2 is a ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound #344 i.e. compound (72) and its numerically outputted data. This data assigned as compound #344 as follows: δ 0.53 (3 H, s), 0.85 (3 H, d, J = 6.7 Hz), 1.08 (3 H, d, J = 6.8 Hz), 1.21 (6 H, s), 2.23 (1 H, dd, J = 13.4, 7.9 Hz), 2.67 (1 H, dd, J = 13.4, 4.0 Hz), 2.83 (1 H, m), 3.83 (1 H, ddd, J = 7.9, 4.4, 4.0 Hz), 4.29 (1 H, d, J = 3.3 Hz), 5.01 (1 H, d, J = 1.8 Hz), 5.28 (1 H, m), 6.01 (1 H, d, J = 11.3 Hz), 6.39 (1 H, d, J = 11.3 Hz).

Note 3 is dated prior to March 17, 1997. The 1st through 3rd pages show the employed protocol for VDR binding assay.

The 1st page shows the 1st to the 8th steps of the protocol. The 1st, I made phosphate buffer and kept it at 4 °C. The 2nd, I prepared diluted solutions of 1α,25(OH)$_2$VD$_3$, compound #344 and compound #346. The 3rd, I prepared [26,27-methyl^{3}H]1α,25(OH)$_2$VD$_3$ solution, took 100 μl of the solution and evaporated, then I added 6.25 ml of Japanese pharmacopeia grade ethanol to the solution. The 4th, I

poured 50 µl of the sample in Japanese pharmacopeia grade ethanol (made in the 2nd step) into disposable culture tubes (12 x 75 mm, product of IWAKI) in order of concentration from thin to dense by dispenser (for example from tube number 14, 28 to 1, 15). Into tubes from number 85 to 96 was poured Japanese pharmacopeia grade ethanol only. The 5th, I made a receptor solution (lot 110431, product of YAMASA). I poured 5 ml of phosphate-potassium buffer (made in the 1st step) into a vessel containing the thymus receptor, dissolved the receptor gently, added further 50 ml of the buffer and stirred gently. The 6th, I added 500 µl of the receptor solution to each tube except blank tubes (tube number 89, 90, 91, 92), and I added 500 µl of the buffer solution to blank tubes. The 7th, I stirred solutions in the tubes (made in the 6th step) by vortex without foaming. The 8th, I pre-incubated the solutions in the tubes at room temperature (approximately 22 °C) for 1 hour (13:40 - 14:40). The tops of the tubes were sealed with plastic wrap and aluminum foil.

The 2nd page shows the 9th to the 11th steps of the protocol. These steps were carried out in RI (radio isotope) room. The 9th, I added 50 µl of the radioactive solution (made in the 3rd step) to each tube by dispenser. In the case of radioactivity only count (tube number 97, 98, 99, 100), radioactive solution was added to the vial. The 10th, I stirred the solution in the tubes by vortex without foaming. The 11th, I sealed the top of the tubes with plastic wrap, put the tubes in a 4 °C refrigerator in the RI room, and stood the tubes overnight from 15:10 (ten after three PM). Then I added 10 ml of ACS-II (Aqueous counting scintillant, product of Amersham) to the tubes and measured radioactivity count for 1 min by Aloka A machine (Scintillation counter). Then I stood the tubes at rt, and the next day I measured the radioactivity count for 2 min with other samples. The data measured for 1 min is shown at the middle of this page. "97 16217.7 dpm" means radioactivity count of tube number 97 was 16217.7 disintegrations per minute. "Average 16370 dpm" means calculated average value of tubes 97, 98, 99, 100 counts which are radioactive only solution. "Average 45 dpm" means calculated average value of tubes 101, 102, 103, 104

counts which are blank solution. In the parenthesis at the bottom of this page there is a calculation of the [26,27-methyl^{3}H]1α,25(OH)$_2$VD$_3$ weight per tube. 16370 dpm is 16370/60 = 273 dps (disintegrations per second), 273 dps is 273 Bq (dps unit equals Bq - becquerel- unit). The radioactivity of the [26,27-methyl^{3}H]1α,25(OH)$_2$VD$_3$ solution used in the 3rd step was 11.4 GBq/mg, (= 11.4 Bq/pg,), so the amount of [26,27-methyl^{3}H]1α,25(OH)$_2$VD$_3$ was 273/11.4 = 24 pg / tube.

The 3rd page shows from the 12th to the 17th steps of the protocol. I carried out these steps the day after the previous steps were done. The 12th, I took the previous day's samples from the refrigerator in the RI room and added 200 µl of DCC (dextran coated charcoal) solution (lot M602 product of YAMASA) to each tube by dispenser except total count tubes (tube number 93, 94, 95, 96). And I added 200 µl of buffer (made in the 1st step) to each of the total count tubes. The 13th, I stirred the tubes by vortex. The 14th, I stood the tubes for 30 min (9:50 - 10:20) at 4 °C. The 15th, the tubes were centrifuged at 3000 rpm for 10 min (10:30 - 10:40) at 0 °C. The 16th, I transferred 500 µl of supernatant of each tube to 20 ml WERATON vial in concentration order from thin to dense (for example from tube number 1 to 14 by same pipetter tip, changed pipetter tip, then from tube number 15 to 28). At that time the vials were put on tray with ice. The 17th, I added 9.5 ml of ACS-II to each of the tubes, shook them and measured its radioactivity count (2min) by Aloka A machine.

The 4th page shows a graph of the radioactivity count (Y-axis) of compounds #346, #344 and 1α,25(OH)$_2$VD$_3$ (control) solutions at various concentration (X-axis) at the 11th step of the assay protocol. Closed circles (VD) and closed triangle (VD-2) show 1α,25(OH)$_2$VD$_3$ values, open circles (344) and crosses (344-2) show #344 compound values and closed diamonds (346) show #346 compound values. These data were measured by Aloka C machine for 1 min. I measured the data from tube number 1 to 70.

The 5th page shows a table of the radioactivity count (dpm) of the compounds #346, #344 and 1α,25(OH)$_2$VD$_3$ (control) solutions at various concentrations. The first column shows the compound's concentration, the 2nd and 3rd columns show the radioactivity counts of 1α,25(OH)$_2$VD$_3$ (duplicate experiment), the 4th and 5th columns show the radioactivity counts of #344 compound (duplicate experiment) and the 6th and 7th columns show the radioactivity counts of #346 compound (duplicate experiment). The small numerals on the line show tube number.

The 6th page shows a table of the radioactivity count of control solutions. "0" in the 1st column means the concentration of the compound is 0, "blank" right below "0" means buffer solution (made in the 1st step of the assay protocol) only, "total count" means radioactive solution (made in the 3rd step of the assay protocol) in the assay tube, "added amount" means added radioactive solution (made in the 3rd step of the assay protocol) and "blank" right below "added amount" means empty tubes. These data was taken by 4 tubes each. The most right column shows the average of the 4 values. The sentence at the middle of this page describes how to calculate the Bound [%] values. The "Bound [%]" means binding percentage of radioactive ligand ([26,27-methyl^{3}H]1α,25(OH)2VD3) to Vitamin D receptor.

The 7th page shows a graph and a table of the radioactivity count of compounds #346, #344 and 1α,25(OH)$_2$VD$_3$ (control) solutions at various concentrations at the last step of the assay protocol. The graph is shown in the same format as the graph on the 4th page, and the table is shown in the same format as the table on the 5th page.

The 8th page shows the method for calculating the binding ratio from the radioactivity data. First I calculated the average values of "blank" and "0" (218 and 2980). Then I described how to calculate the Bound [%] values. In the open bracket, I calculated the radioactive ligand ([26,27-methyl^{3}H]1α,25(OH)$_2$VD$_3$) amount in the assay tubes from "total count" values (19 pg / tube). Then I

calculated added radioactive ligand from "added amount" values (24 pg / tube). Then I speculated as to why there was a difference between the values for the "total count" and the "added amount."

The 9th page shows a graph and a table of the binding ratio of compounds #346, #344 and $1\alpha,25(OH)_2VD_3$ (control) at various concentrations. The binding ratio was calculated by the method described in the 8th page. In the graph, the concentration value (X-axis) of the compounds #346, #344 and $1\alpha,25(OH)_2VD_3$ at 50% bound (Y-axis) are 10-20 pg, 1-2 pg and 20 pg. These data show the binding affinity of compound #344 / #346 is 10-12 / 1-1.8 fold stronger than that of $1\alpha,25(OH)_2VD_3$.

Chart 3 is dated prior to March 17, 1997. Chart 3 is an output of the liquid scintillation counter mentioned on the 3rd page of Note 3. The data were transferred to the 5th and 6th pages of Note 3. The upper half side of the 1st page shows the settings of the scintillation counter. The bottom side of the 1st page and 2nd page shows raw data. The most left column shows tube number, the 3rd column shows the period of measuring (2 min) and the 5th column shows the radioactivity count (unit is dpm).

Exhibit 2 is a set of copies of handouts that I distributed at the group seminar referred to above. The handouts consist of 2 parts. As described at the top of the 1st page of the handout the first part relates to Synthesis of 2-methyl-20epi $1\alpha,25(OH)_2VD_3$.

Page 1 shows the synthesis scheme for compounds #346 and #344, i.e. compounds (68) and (72) respectively. The 1st arrow pointing from vitamin D_2 to compound 1 indicates that I made compound 1 from vitamin D_2 by treatment of vitamin D_2 with O_3 (Ozone) and $NaBH_4$ (Sodium borohydride). The 2nd arrow pointing from compound 1 to compound 2 indicates that I made compound 2 from compound 1 by treatment of compound 1 with TsCl (Tosyl chrolide) in pyridine (Yield 86%). The 3rd arrow pointing from compound 2 to compound 3 indicates that I made compound 3 from compound 2 by treatment of compound 2 with TBSOTf (tert-Butyldimethylsilyl triflate) in 2,6-lutidine (Yield 96%). The 4th arrow pointing from compound 3 to compound 4 indicates that I made compound 4 from compound 3 by

treatment of compound 3 with DMSO (Dimethylsulphoxide) and $NaHCO_3$ (Sodium bicarbonate) (Yield 76%). The 5th arrow pointing from compound 4 to compound in parenthesis indicates that I made the compound in parenthesis from compound 4 by treatment of compound 4 with n-Bu_4NOH (normal-Tetrabutylammonium hydroxide) in CH_2Cl_2 (Dichloromethane) and H_2O (water). The 6th arrow pointing from the compound in parenthesis to compound 5 indicates that I made compound 5 from the compound in parenthesis by treatment of the compound in parenthesis with $NaBH_4$ (Sodium borohydride) and stereoisomer separation by silica gel column chromatography (Yield 45% in 2 steps). The 7th arrow pointing from compound 5 to compound 6 indicates that I made compound 6 from compound 5 by treatment of compound 5 with TsCl (Tosyl chrolide) in pyridine (Yield 93%). The 8th arrow pointing from compound 6 to compound 7 indicates that I made compound 7 from compound 6 by treatment of compound 6 with NaI (Sodium iodide) in DMF (Dimethyl formamide) (Yield 92%). The 9th arrow pointing from compound 7 to compound 8 indicates that I made compound 8 from compound 7 and the compound described above the arrow by treatment of both compounds with n-BuLi (normal-Butyl lithium) and HMPA (Hexamethylphosphoramide) in THF (Tetrahydrofuran) (Yield 72% with recovered 28% of starting compound 7. The yield of this step was increased when using distilled HMPA --- 99%). The 10th arrow pointing from compound 8 to compound 9 indicates that I made compound 9 from compound 8 by treatment of compound 8 with Na-Hg (sodium-mercury amalgam) in THF (Tetrahydrofuran) (Yield 64%). The 11th arrow pointing from compound 9 to compound 10 indicates that I made compound 10 from compound 9 by treatment of compound 9 with TsOH (p-Toluenesulfonic acid) (Yield 85%). The 12th arrow pointing from compound 10 to compound 11 indicates that I made compound 11 from compound 10 by treatment of compound 10 with TPAP (Tetrapropylammonium perruthenate), NMO (N-methylmorpholine) and 4ÅMS (molecular sieves 4 angstrom) (Yield 87%). The 13th arrow pointing from compound 11 to compound 12 indicates that I made compound 12 from compound 11 by treatment of compound 11 with $Ph_3P(+)CH_2Br \cdot Br(-)$

((Bromomethyl)triphenylphosphonium bromide) and NaHDMS (Sodium hexamethyldisilazide) (Yield 57%). The 14th and 15th arrows pointing from compound 12 to 20epi-Ds (compound #344) and 20epi-Aa (compound #346) indicate that I made 20epi-Ds and 20epi-Aa from compound 12 and a TBS protected compound (described in the upper of the page 5 and 6 of the Exhibit 2) by treatment of both compounds with $(dba)_3Pd_2CHCl_3$, (Tris(dibenzylideneacetone)dipalladium(0)-chloroform adduct), Ph_3P (Triphenylphosphine) and Et_3N (Triethylamine) in toluene, and then treatment of the resultant compounds with CSA (Camphor sulfonic acid) in MeOH (methanol).

Page 2 shows the method for the VDR binding assay. The resultant binding curves of compounds #346, # 344 and $1\alpha,25(OH)_2VD_3$ (control) are also shown. The upper half of the page shows the method for VDR binding assay which has the same content as the description in the 1st through 3rd pages of Note 3. The bottom half side of the page shows the resultant binding curves which is the same graph described on the 9th page of Note 3.

Pages 3 through 6 show the detailed process for synthesizing compounds #346 and #344 from compound 7 as described in scheme 1 on page 1, accompanied by ^1H NMR, MS and HRMS data of each intermediate compound. As for compounds #346 and #344 i.e. compounds (68) and (72), UV data are also shown. Page 3 shows the detailed process for synthesizing compound 8 from compound 7 and compound 9 from compound 8. Page 4 shows the detailed process for synthesizing compound 10 from compound 9, compound 11 from compound 10 and compound 12 from compound 11. Page 5 shows the detailed process for synthesizing 20epiDs (compound #344) from compound 12 and a TBS protected compound. Page 6 shows the detailed process for synthesizing 20epiAa (compound #346) from compound 12 and a TBS protected compound.

Exhibit 3 is a copy of the abstract that I submitted for a poster session at the Tenth Workshop on Vitamin D. Attached to the abstract is a copy of the instructions for submitting the abstract.

The abstract confirms that I had designed and synthesized the 2-methyl-20-epi analogues of 1α,25-dihydroxyvitamin D_3 and had determined the activity of the analogues. In the abstract, compounds (1) and (2) correspond to compounds (72) and (68) in the specification of the above-identified application.

The instructions confirm that the abstract had to be submitted prior to March 17, 1997.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: _____

_____
Toshie Fujishima